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"MOCK FISH" METHOD FOR STUDYING MICROBIAL INHIBITING AGENTS

In experiments intended to study the effects of various agents or conditions on the microbial outgrowth in food products, it is desirable to approach efficacy similar to those conditions of actual handling and marketing. However, in experiments on fishery products, when one wishes to find effects of an agent or condition, the use of whole fish or fish fillets adds variables to any experimental design. These undesired variables are: variations in the total microbial population and in the composition of the microbial flora from fish to fish; different time intervals and other storage variations in the handling history of fish even from the same catch; different fillet or sample thicknesses which will affect the counts per gram ratio from sample to sample; different physiological conditions, age, wounds, etc., of the fish which might affect experimental comparisons; and possible presence of inherent antibiotics in the substrate. The latter variable does not permit a separation of the antibiotic effects of the additives from the antibiotic effects of the substrate.

In order to study what effects agents might actually have on specific microbial outgrowth in an efficacious situation, a "mock fish," composed of gelatin (containing nutrients) and supported structurally with cheesecloth was devised. The mock fish allowed us to control: total number and composition of the microbial flora; location of microbial contamination, e.g., surface or evenly dispersed throughout the sample; uniformity of distribution of microbes from sample to sample; size and thickness of the samples; and the handling history and physiological state of the samples. This system permits the quantitative recovery of the inoculated microbes by simply melting the gelatin at 31°–32° C.

This note describes the application of mock fish in studying the effects of disodium ethylenediamine tetraacetate (EDTA, Fisher Scientific Co.[1]) with or without an iodophor (Wyandotte Co.) contained in ice for controlling microbial outgrowth of a mixture of four *Pseudomonas* species. This procedure is not recommended as a means of predicting the effectiveness of an inhibitor on a specific species of fish. Its role is to screen inhibiting agents for general effectiveness and to permit a comparison among them.

Materials and Methods

Mixture of *Pseudomonas* Species

Four *Pseudomonas* species, previously isolated from iced fish in our laboratory, were used in these experiments. Each species of *Pseudomonas* was grown in separate Eugon Broth (BBL) test tube culture for 18 h at 20°C. Then 2 ml from each culture were pooled and well mixed in a sterile test tube to prepare an inoculum mixture. From this mixture 1 ml was inoculated into 1 liter of melted gelatin medium described below to give an estimated 10^4 to 10^5 bacteria/ml of the final preparation.

Mock Fish Preparation

1) Cheesecloth discs were cut to size to fit inside glass Petri dishes, and then they were cut in half. The Petri dishes were then sterilized at 121°C for 15 min.

2) Ten milliliters of melted, inoculated 10% gelatin and 1% Eugon Broth medium were pipetted into each sterile Petri dish. A sterile needle was used to make sure that the cheesecloth disc halves did not overlap during gelatin solidifica-

[1]Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

tion. Once solidified, the gelatin in each plate was cut in half with a sterile needle along the cheesecloth division, thus making two mock fish for use in experimental procedures. The mock fish were gently pried loose from the Petri dish with the aid of a sterile spatula or large, blunt forceps and placed into a beaker containing crushed ice. The cheesecloth provides ample structural support to the solidified gelatin.

Preparation of Crushed Ice

To minimize contamination, distilled water, glassware, ice cube trays, and an ice cube crusher were sterilized prior to use in the preparation of solutions and crushed ice. Using distilled water to minimize the presence of chlorine, minerals, etc., the following solutions were prepared: 1) 1% EDTA; 2) 0.1% EDTA; 3) 1% EDTA plus 1% $CaCl^2$; 4) 0.1% EDTA plus 1% CaCl; and 5) 0.1% EDTA plus 10 ppm of Accord (an iodophore manufactured by BASF Wyandotte Corp., Wyandotte, Mich.).

In order to demonstrate the applicability of this mock fish method, we tested the effect of EDTA embedded in ice on typical *Pseudomonas* species found associated with iced fish. Interest in EDTA for use as a microbial inhibitor has been cited by Levin (1967), Winarino et al. (1971), and Maunder et al.[2] The addition of calcium ions was to interfere with the chelating property of EDTA. The addition of an iodophor was to observe for a possible greater effect.

The control ice contained no added ingredients. These solutions were poured into ice cube trays and frozen. A hand operated individual ice cube crusher was used to prepare crushed ice to fill 800-ml beakers. From 8 to 10 mock fish were placed into each beaker containing crushed ice and stored at 0°C for the duration of the experiment.

Bacterial Assays

At each time interval (0, 1, 3, 6, and 11 days), mock fish were removed from each beaker and placed in a sterile plastic petri dish. The Petri dishes were floated on a 31°–32°C water bath to melt the gelatin. Aliquots of the melted, well-stirred gelatin were decimally diluted and plated using Eugon Agar (BBL) with 0.1% yeast extract (BBL) added. Plates were incubated at 20°C for 5 days prior to counting.

Results and Discussion

The results of the experiments are shown in Figure 1. The initial starting population was 4.5×10^4 pseudomonads/ml of gelatin medium. The resulting growth patterns reflect the effect of agents contained in the ice and melt water. By the 5th day, melt water entirely surrounded the mock fish in each beaker. By about the 10th day, the floating ice composed one-half to one-third of the beaker contents.

The mock fish held together throughout the experiment with only occasional slivers, not supported by the cheesecloth, breaking off.

The mock fish method permits an evaluation of the effects of microbial inhibiting additives, used singly or in combination, to yield relatively accurate results. Thus, the method may be used to screen a wide variety of antibiotic systems before going into efficacy studies. The value of the mock fish system is that it not only permits a broad screening of additives, but it also permits one to determine, in efficacy studies, whether microbial inhibition is due to additives alone or partly to substrate antibiotic components such as certain polypeptides (J. T. R. Nickerson pers. commun.). It affords a method of controlling some variables and/or allowing the study of effects upon specific microorganisms. We have employed versions of

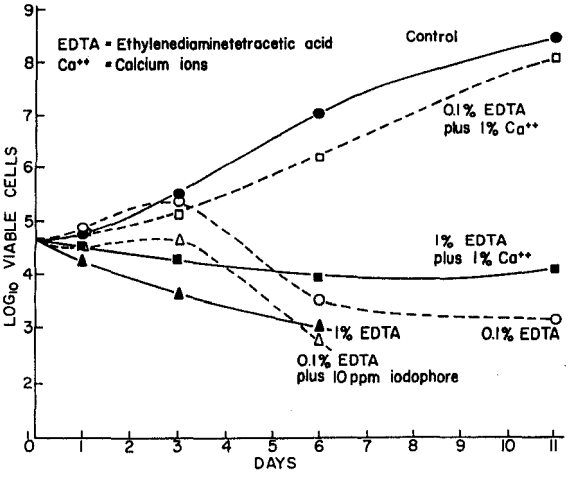

FIGURE 1.—Survival of *Pseudomonas* spp. in mock fish.

[2]Maunder, D. T., W. P. Segner, C. F. Schmidt, and J. K. Boltz. 1966. Growth characteristics of Type E *Clostridium botulinum* in the temperature range of 34 to 50°F. Annu. Rep. to U.S. At. Energy Comm. (now ERDA), Contract No. AT(11-1)1183.

mock fish before in irradiation studies in which we either embedded the inoculum evenly throughout the gelatin disc or smeared the same size inoculum on one surface of the gelatin disc (Green and Kaylor 1977). The method might be extended to other applications where some detail or specific effects are to be elucidated.

From Figure 1 it is obvious that 1% calcium ions negate the effect of 0.1% EDTA and reduce the effect of 1% EDTA. An improved effect is noticed when 10 ppm iodophor is coupled with 0.1% EDTA, and this was somewhat expected.

The implied conclusion is that 1% EDTA embedded in ice, free of divalent ions, will reduce the outgrowth of *Pseudomonas* spoilage organisms on iced fish and that the inhibitory effect of 0.1% EDTA combined with 10 ppm iodophor is even greater. The expected results obtained with the mock fish supports their reliability for the intended use, but it is not suggested for use as a substitute for efficacy tests. Therefore, conculsions regarding the effectiveness of inhibitory additives for any specific substrate must ultimately be derived from conventional efficacy tests.

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REPRODUCTIVE CYCLE OF THE PINK SURFPERCH, *ZALEMBIUS ROSACEUS* (EMBIOTOCIDAE)

Embiotocids received early attention from biologists (e.g., Eigenmann 1892) partly because of the viviparous mode of reproduction displayed by fishes of this family. The pink surfperch, *Zalembius rosaceus* (Jordan and Gilbert), is one of the lesser known members of this group. What is most distinctive about *Z. rosaceus* as compared with other embiotocids is the timing of the various events of its annual reproductive cycle. The purpose of this report is to describe this cycle.

Materials and Methods

Specimens were collected off the coast of southern California at depths ranging from 27 to 33 m. Samples were taken from Redondo Beach, Los Angeles County, to San Clemente, Orange County, Calif. Monthly collections were obtained from May 1972 to September 1973 and January and March 1977. Collections were made using otter trawls from the Occidental College RV *Vantuna* and from the RV *Fury II*, operated by the Orange County Board of Education. Specimens from July, August, and September 1973 were provided by the Southern California Coastal Water Research Project. Specimens were also examined in the ichthyology collection of the Los Angeles County Museum of Natural History.

The fish were preserved in 10% Formalin.[1] Gonads were embedded in paraffin. Histological sections were cut at 8 μm and stained with iron hematoxylin followed by eosin counterstain. Gonads were sectioned from the following numbers of females: January (7), February (4), March (11), April (6), May (5), June (10), July (1), August (15), September (18); October (3); December (6); and from 85 males, as shown in Table 1. Sectioned material was collected in 1973 except that for May, June, October, and December 1972.

Results and Discussion

The gonadal morphology and histology of *Z. rosaceus* closely resembles that of the embiotocids *Cymatogaster aggregata* as described by Eigen-

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